Role of Na^+/Ca^{2+} exchange in regulation of neuronal Ca^{2+} homeostasis requires re-evaluation

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Abstract In cultured rat cerebellar granule cells an inhibition of plasma membrane Na^+/Ca^{2+} exchange by removal of external Na^+ (replacement with NMDG) caused an increase in $[Ca^{2+}]_i$ at rest and a considerable delay in $[Ca^{2+}]_i$ recovery from Glu-imposed $[Ca^{2+}]_i$ load. These effects did not result from Ca^{2+} influx through reversed Na^+/Ca^{2+} exchange since they were readily abolished or prevented by using the NMDA receptor inhibitor AP-5 (100 μM) or the NMDA channel blocker memantine (25–50 μM). The effect of Na^+/NMDG replacement could be enhanced by: (1) an increase in cytoplasmic Na^+ concentration by monensin pretreatment of neurons; (2) external alkalinity, pH 8.5; (3) blockade of the mitochondrial Ca^{2+} uptake with antimycin plus oligomycin. Analysis of the data obtained led us to conclude that all the changes in $[Ca^{2+}]_i$ caused by Na^+/NMDG replacement are mainly due to a release of endogenous Glu (reversed Glu uptake) and a subsequent Ca^{2+} influx through NMDA receptor-mediated channels.

Key words: Neurotoxicity; Glutamate; Na^+/Ca^{2+} exchange; Cultured neuron

1. Introduction

At present, it is widely accepted that the plasma membrane Na^+/Ca^{2+} exchanger plays a key role in the regulation of Ca^{2+} homeostasis in most living cells, including mammalian central neurons [1,2]. Thus, both in cultured hippocampal neurons [3,4] and in cerebellar granule cells [5] the replacement of external Na^+ (Na^+_o) with organic cations, in particular *N*-methyl-D-glucamine (NMDG), causes a dramatic deterioration of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) recovery following a short-duration glutamate (Glu) challenge. This led the authors to conclude that the post-glutamate $[Ca^{2+}]_i$ recovery is mediated mainly by the Na^+/Ca^{2+} exchange system in the neuronal membrane. It is known, however, that in mammalian central neurons and glial cells lowering of the transmembrane Na^+ gradient reverses not only the Na^+/Ca^{2+} exchange but also the mode of operation of the plasma membrane glutamate carrier [6], which may lead to release of endogenous Glu (reversed uptake) with a subsequent activation of *N*-methyl-D-aspartate (NMDA) receptors and Ca^{2+} influx into the neuron [7].

The objective of our work was to evaluate the possible contribution of the endogenous Glu release to changes in $[Ca^{2+}]_i$ induced by Na^+/NMDG replacement. To this end we used a specific competitive antagonist of NMDA receptors, AP-5, and a direct blocker of open NMDA channels, memantine (Mem). Unexpectedly we found that most of the changes in $[Ca^{2+}]_i$ evoked by Na^+_o removal under various experimental conditions could be almost completely blocked by these NMDA antagonists. Thus, the role of the Na^+/Ca^{2+} exchanger in regulation of Ca^{2+} homeostasis should be re-evaluated.

2. Materials and methods

Dissociated cerebellar granule cell cultures were prepared from the cerebella of 7–8 day old Wistar rats using the procedure described in [8]. The cells were grown in MEM (Sigma) containing: 10% fetal bovine serum, 10 mM HEPES, 12 mM $NaHCO_3$, 2 mM L-glutamine, 0.2 U/ml insulin, 0.6% glucose, 25 mM KCl (added on day 3) and 2.5 μM Ara-C (36.5°C, +5% CO_2). The experiments were carried out on 7–9 day cell cultures. $[Ca^{2+}]_i$ and intracellular pH (pH_i) were measured in neurons loaded with the fluorescent dyes FURA-2/AM (5 μM) or BCECF/AM (10 μM) for 1 h at 37°C in the medium mentioned above. Cells were then washed with a control HEPES-buffered salt solution (HBSS) and placed to an experimental chamber. The chamber was mounted on a Nikon inverted-stage microscope connected with a SPEX spectrofluorimeter (New Jersey, USA) equipped with a dual mirror chopping mechanism with a specialized optical configuration to allow rapid alteration (100 Hz) between two excitation wavelengths. Fluorescence of the Ca^{2+} sensitive dye FURA-2 was measured using excitation wavelengths of 340 nm and 380 nm and an emission filter at 510 nm. The buffers for $[Ca^{2+}]_i$ calibration were prepared as described in [5]. Fluorescence of the H^+ sensitive dye BCECF was measured using excitation wavelength of 488 nm and emission filter at 535 nm. The buffer for pH_i calibration consisted of (mM): 10 NaCl, 100 K-gluconate, 1 $MgCl_2$, 20 HEPES and 25 μM K^+/H^+ ionophore nigericin. The HBSS contained (mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5 glucose, 10 sucrose, pH 7.4. In a Na^+-free solution NaCl was replaced with 140 mM NMDG (pH was adjusted with 8.5 mM HCl).

Cell survival was assessed by counting living neurons using trypan blue exclusion staining [9]. FURA-2/AM and BCECF/AM were purchased from Molecular Probes (USA). All the other chemicals were obtained from Sigma or Fisher Chemicals (USA).

3. Results and discussion

3.1. Effect of reduction of the transmembrane Na^+ gradient

3.1.1. Removal of external Na^+ (Na^+_o). In most of the

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Abbreviations: $[Ca^{2+}]_i$ and $[Na^+]_i$, cytosolic Ca^{2+} and Na^+ concentrations; FURA-2 AM, FURA-2 acetoxymethyl ester; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; Ara-C, cytosine arabinoside; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; (+/−)-CPP, (+/−)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; NMDA, *N*-methyl-D-aspartate; Glu, glutamate; AP-5, 2-amino-5-phosphonopentanoic acid; Mem, memantine; Mon, monensin; NMDG, *N*-methyl-D-glucamine; HBSS, HEPES-buffered salt solution; CaR, $[Ca^{2+}]_i$ response

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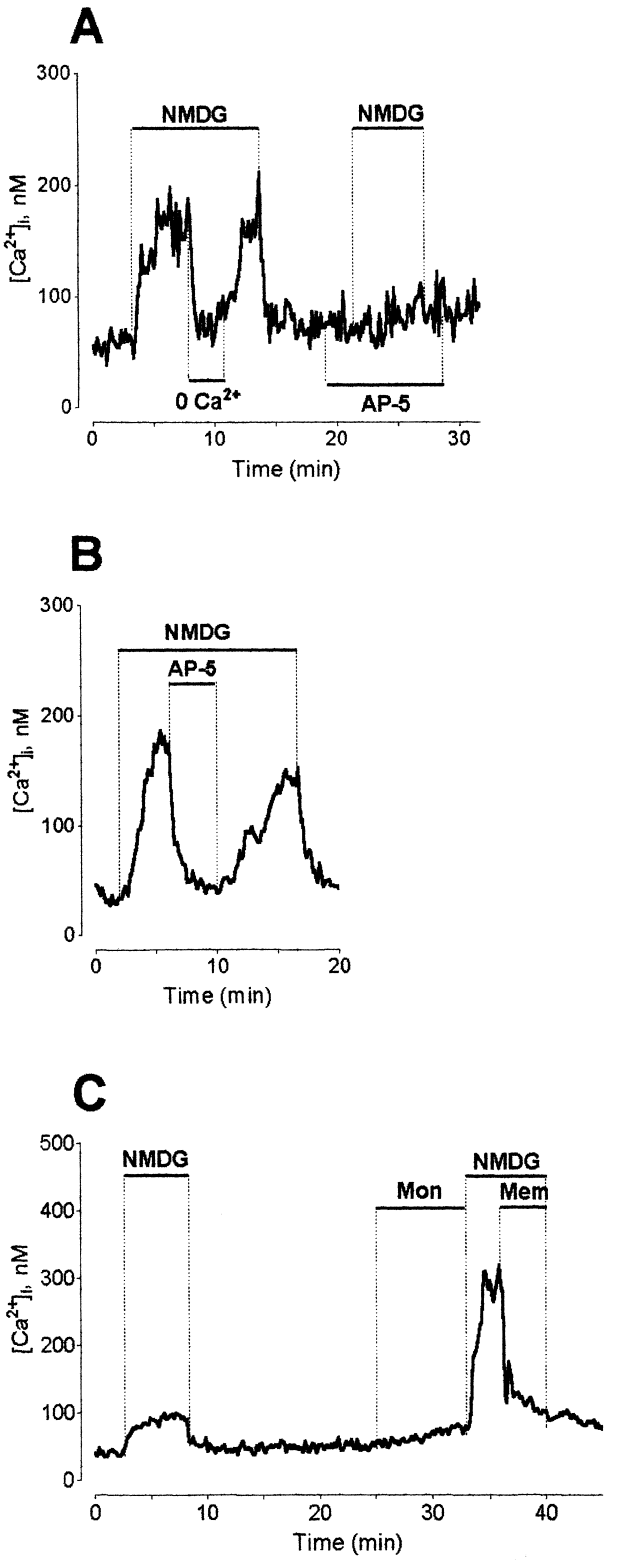

Fig. 1. A,B: Ca^{2+} responses caused by Na^+/NMDG replacement were blocked either by removal of external Ca^{2+} or by 100 μM AP-5 (representative of seven experiments). C: Ca^{2+} responses caused by Na^+/NMDG replacement were enhanced by increasing $[Na^+]_i$ and blocked by 50 μM memantine. To increase $[Na^+]_i$, the nerve cells were pretreated by monensin (representative of six experiments). NMDG, solution in which Na^+ was completely replaced by NMDG; $-Ca^{2+}$, nominally Ca^{2+}-free NMDG-containing solution; AP-5, NMDA receptor inhibitor; Mem, memantine; Mon, monensin.

cerebellar granule cells used in this study, replacement of Na^+ by NMDG in the control HBSS solution induced a reversible increase in $[Ca^{2+}]_i$. The magnitude of this $[Ca^{2+}]_i$ response (CaR) varied between the cells over a wide range (Fig. 1). Removal of external Ca^{2+} prevented or abolished CaR (Fig. 1A) indicating its dependence on the Ca^{2+} influx. However, this influx cannot be explained by an unmasking or enhancement of the Na^+_i/Ca^{2+}_o exchange caused by removal of Na^+_o, since the CaR was effectively prevented or inhibited by addition of 100 μM AP-5 to the Na^+-free solution (Fig. 1A,B).

3.1.2. Increase in cytoplasmic Na^+ ($[Na^+]_i$). An increase in $[Na^+]_i$ is known to enhance the exchange of Na^+_i for Ca^{2+}_o. In order to increase $[Na^+]_i$ we used the ionophore monensin (Mon, 10 μM). In Fig. 1C the Na^+/NMDG replacement before the Mon application produced only a very low CaR. Pretreatment of the cell with Mon (7 min) also caused only a slow and small increase in $[Ca^{2+}]_i$ by itself, however, the subsequent Na^+/NMDG replacement evoked a prominent $[Ca^{2+}]_i$ elevation. This effect could not be explained by exchange of high Na^+_i for Ca^{2+}_o since the addition of Mem (50 μM) abolished this CaR almost completely. Qualitatively similar results were obtained in case of monensin and AP-5 coapplication (not illustrated).

3.2. Effect of external pH_o elevation

Fig. 2A shows that an increase in pH_o from 7.4 to 8.5 caused a small elevation of the basal $[Ca^{2+}]_i$ and a considerable increase in the CaR to Na^+/NMDG replacement. Addition of AP-5 abolished the effect of Na^+_o removal indicating that at high pH_o, as well as at pH_o 7.4, the CaR mainly resulted from the Ca^{2+} influx via NMDA channels. An increase in the amplitude of the CaR at pH_o 8.5 can be readily explained by the fact that pH_o elevation potentiated the agonist-induced activation of NMDA channels [10] along with suppression of the Ca^{2+}/H^+ pump-mediated Ca^{2+} extrusion from the cell [11]. Note that AP-5 did not prevent $[Ca^{2+}]_i$ elevation induced by high pH_o.

3.3. Block of Ca^{2+} uptake by mitochondria

In order to block the mitochondrial electrophoretic Ca^{2+} uptake [12] the cells were treated with a cocktail of 0.5 μM antimycin, the inhibitor of mitochondrial respiration, and 2.5 μg/ml oligomycin, an inhibitor of the mitochondrial ATP-ase which produced a collapse of the mitochondrial potential [13]. Fig. 2B shows that in the presence of mitochondrial poisons the CaR to Na^+/NMDG replacement was enhanced as compared to the control value measured before mitochondrial depolarization. Addition of 100 μM AP-5 suppressed this effect indicating that the blockade of mitochondrial Ca^{2+} uptake increased CaR induced by endogenous Glu (but not by Na^+_i/Ca^{2+}_o exchange).

3.4. Effects of Na^+/NMDG replacement on $[Ca^{2+}]_i$ recovery following a Glu pulse

In agreement with previous reports [3–5], in our experiments the Na^+/NMDG replacement just after the termination of a 1-min Glu pulse greatly delayed the $[Ca^{2+}]_i$ recovery. Addition of 100 μM AP-5 to the Na^+-free post-glutamate solution effectively eliminated this delay: thus in Fig. 3A the time course of $[Ca^{2+}]_i$ recovery following a 1-min Glu pulse in a NMDG and an AP-5 containing solution did not differ from that in the control medium. Evidently the delay in the post-

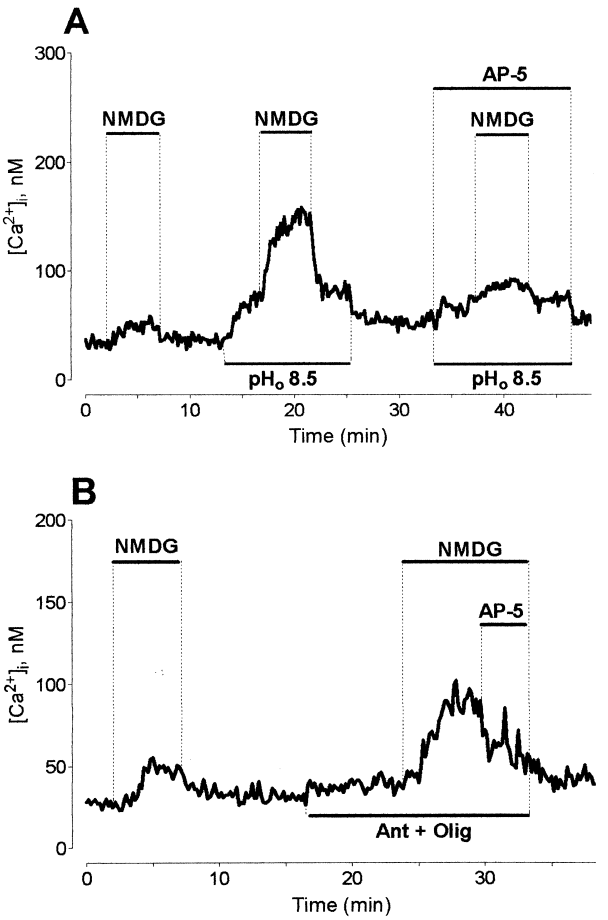

Fig. 2. Blockade by AP-5 of Ca^{2+} responses caused by Na^+/NMDG replacement at pH_o 8.5 (A, representative of five cells) and under the action of mitochondrial poisons (B, representative of three cells): Ant+Olig, mixture of antimycin (0.5 μM) and oligomycin (2.5 μg/ml).

glutamate $[Ca^{2+}]_i$ recovery resulted from Ca^{2+} influx via NMDA channels activated by endogenous Glu.

3.5. Changes in cytoplasmic pH_i

Na^+/NMDG replacement is known to induce cytoplasmic acidification due to the turning off of the plasmalemmal $Na^+{}_o$/$H^+{}_i$ exchange. Koch and Barish [3] observed this effect in cultured hippocampal neurons and believed that such a decrease in pH_i makes a considerable contribution to the mechanism of the slowing down of the post-glutamate $[Ca^{2+}]_i$ recovery caused by Na^+/NMDG replacement. Fig. 3B shows that the Na^+/NMDG exchange caused a reversible pH_i reduction both at rest and following a Glu pulse. It is seen also that in both cases AP-5 application failed to attenuate these changes in pH_i, although the changes in $[Ca^{2+}]_i$ produced by Na^+/NMDG replacement under the same conditions were practically completely abolished. Evidently, pH_i reduction caused by $Na^+{}_o$ removal did not depend appreciably on a concomitant increase in $[Ca^{2+}]_i$.

3.6. Cell viability

In parallel experiments on sister cultures, we assessed the effect of Na^+/NMDG replacement on cell viability. A 30-min bathing of the cell culture in the control solution in which Na^+ was replaced with NMDG produced a three-fold increase in the proportion of dead cells counted 4 h after the above treatment. For a comparison Fig. 4 presents the toxic effect of 30-min treatment of sister cells by 100 μM Glu under similar conditions. Addition of 100 μM AP-5 to a Na^+-free (NMDG) solution induced a pronounced protective effect. This result is in good agreement with that obtained by Takahashi and Hashimoto [7] in experiments with cultured hippocampal slices. In their experiments, external NaCl was partially replaced with choline. When the slices were exposed to 3.6 mM Na^+ for 30 min at 36°C, almost all the neurons in the CA1 region degenerated within 20–24 h. The NMDA channel blockers MK 801 (1–3 μM) and (+/−)-CPP (30 μM) significantly suppressed these neurotoxic effects. In parallel experiments the authors revealed a 40-fold increase in Glu release produced by 30-min exposure to 3 or 6 mM Na^+, which was partly independent of external Ca^{2+}, thus resulting from reversed Glu uptake.

In conclusion, the current notions on the role of Na^+/Ca^{2+}-mediated Ca^{2+} extrusion in regulation of Ca^{2+} homeostasis in mammalian central neurons require revision. These notions are largely based on the erroneous interpretation of the effects of external Na^+ removal on cytoplasmic Ca^{2+} dynamics: the

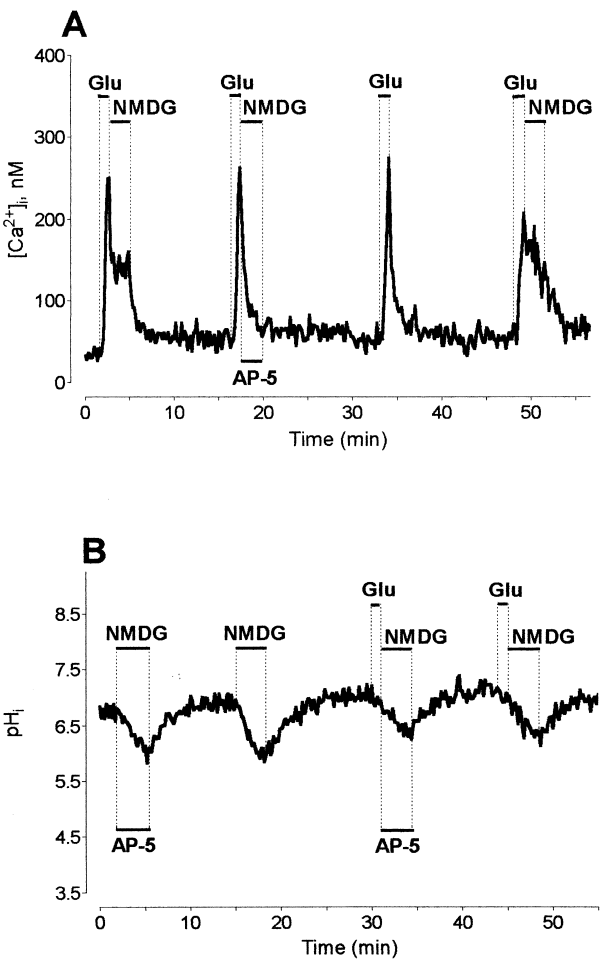

Fig. 3. A: AP-5 removes the delay in $[Ca^{2+}]_i$ recovery induced by Na^+/NMDG replacement following a Glu pulse (representative of six experiments). B: AP-5 does not remove the pH_i decrease-induced Na^+/NMDG replacement either at rest or following a 2-min Glu pulse (representative of three experiments).

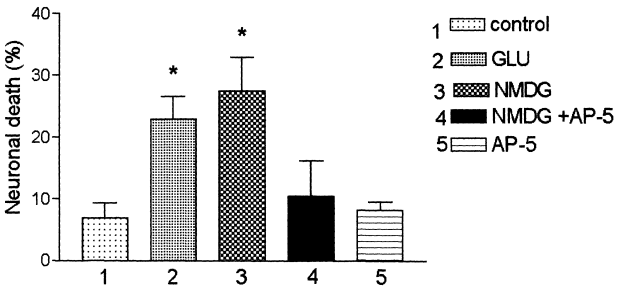

Fig. 4. AP-5 protects nerve cells against death induced by Na^+/NMDG replacement. $^*P < 0.05$ as compared with control HBSS. AP-5, 100 μM; Glu 100 μM. The duration of all the exposures was 30 min (for explanations see text).

authors did not take into account that a replacement of Na^+ with NMDG (or with other organic cation) in the external solution reverses not only the Na^+/Ca^{2+} exchange but also the Na^+-dependent transmembrane Glu transport. Our experiments with cultured cerebellar granule cells show that it is just a release of endogenous Glu (reversed Glu uptake) and a subsequent activation of NMDA receptors, which is responsible for most of the changes in $[Ca^{2+}]_i$ induced by Na^+/NMDG replacement, at rest as in the post-Glu period. The pharmacological blockade of NMDA receptor channels prevents or abolishes these effects of Na^+_o removal indicating that the Na^+_i/Ca^{2+}_o exchange does not play a dominant role in the regulation of neuronal Ca^{2+} homeostasis.

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